The Journal of Experimental Biology 203, 3703–3715 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
JEB3010

FUNCTIONAL UREOGENESIS IN THE GOBIID FISH, *MUGILOGOBIUS ABEI*

KATSUYA IWATA[1,*], MAKIKO KAJIMURA[2] AND TATSUYA SAKAMOTO[3]

[1]*Biological Laboratory, Faculty of Education, Wakayama University, Wakayama 640-8510, Japan,*
[2]*Department of Bio-and Geo-Sciences, Graduate School of Science, Osaka City University, Osaka 558-8585, Japan*
and [3]*Department of Biology, Faculty of Integrated Arts and Sciences, Hiroshima University, Higasi-Hiroshima 739-8512, Japan*

*e-mail: katsuiw@center.wakayama-u.ac.jp

Accepted 25 September; published on WWW 14 November 2000

Summary

To examine the transition to ureogenesis, the gobiid fish *Mugilogobius abei* was immersed in 2 mmol l^{-1} NH_4HCO_3 or a ^{15}N-labelled ammonia solution [1 mmol l^{-1} $(^{15}NH_4)_2SO_4$, pH 8.0] for 4–8 days. When exposed to 2 mmol l^{-1} NH_4HCO_3 or ^{15}N-labelled ammonia solution for 4 days, the rate of urea excretion increased to seven times that of the control (in 20 % synthetic sea water) and remained at this level for 4 days. The proportion of nitrogen excreted as urea reached 62 % of total nitrogen excretion (ammonia-N + urea-N). ^{15}N-enrichment of the amide-N in glutamine in the tissues of fish exposed to ^{15}N-labelled ammonia was virtually the same as that of ammonia-N: i.e. approximately twice that of urea-N in the excreta and the tissues. Glutamine contents and glutamine synthetase activities in the liver and muscle increased greatly following exposure to ammonia. Urea and citrulline contents in the muscle and whole body of the exposed fish increased significantly, whereas uric acid contents remained unchanged. Carbamoyl phosphate synthetase III (CPSase III) mRNA expression and CPSase III activity were detected in the muscle, skin and gill, but levels were negligible in the liver. Furthermore, all other ornithine–urea cycle (O-UC) enzymes were also detected in muscle, skin and gill. Thus, *M. abei* clearly shows the transition from ammoniotely to ureotely under ammonia-loading condition and is able to produce urea mainly *via* the O-UC operating in multiple non-hepatic tissues as a means for ammonia detoxification.

Key words: facultative ureotely, ammonia detoxification, CPSase III mRNA, ornithine–urea cycle, glutamine synthetase, [^{15}N]ammonia, [^{15}N]urea, muscle, skin, gill, gobiid fish, *Mugilogobius abei*.

Introduction

In teleosts, only a few species, such as the Lake Magadi tilapia *Oreochromis alcalikus grahami* (Randall et al., 1989), the gulf toadfish *Opsanus beta* (Walsh et al., 1990; Walsh and Milligan, 1995; Kong et al., 2000) and two species of Indian amphibious catfish, *Heteropneustis fossilis* (Saha and Ratha, 1987, 1994) and *Clarias batrachus* (Saha et al., 1999), are known to produce urea *via* the ornithine–urea cycle (O-UC) in response to adverse environmental conditions such as severely alkaline water, confinement and ammonia loading. The majority of teleosts have non-detectable or very low activities of the key enzymes in the O-UC, in particular of carbamoyl phosphate synthetase III (CPSase III), which catalyzes the first entry of nitrogen into the O-UC (Mommsen and Walsh, 1991; Anderson, 1995; Korsgaard et al., 1995). In both freshwater and marine teleosts, CPSase III activity and CPSase III mRNA are detected during the early life stages (Wright et al., 1995; Korte et al., 1997; Chadwick and Wright, 1999; Terjesen et al., 2000); however, in the adult stage, CPSase III expression is apparently absent or is present at low levels in non-hepatic tissue (e.g. muscle in rainbow trout *Oncorhynchus mykiss* and halibut *Hippoglossus hippoglossus*) (Korte et al., 1997; Terjesen et al., 2000). In several teleosts, including largemouth bass *Micropterus salmoides*, carp *Cyprinus carpio* and bowfin *Amia calva*, low levels of CPSase III and other O-UC enzymes are known to be present in muscle (Kong et al., 1998; Felskie et al., 1998). Despite the expression of O-UC enzymes in these species during the adult stage, it seems unlikely that they have a functional urea-producing ability, i.e. functional ureogenesis. For example, when exposed to elevated external ammonia levels, largemouth bass and bowfin show only a limited enhancement of urea excretion (McKenzie and Randall, 1990; Kong et al., 1998).

Recently, Saha et al. (1999) reported that, in the ureogenic amphibious Indian catfish *Clarias batrachus*, high levels of all O-UC enzymes [except argininosuccinate lyase (ASLase) in muscle and argininosuccinate synthetase (ASSase) in brain] were detected in the liver, kidney, intestine, muscle and brain. Furthermore, in the Lake Magadi tilapia, it has been reported that CPSase III and all other urea cycle enzymes are present in muscle at levels that are sufficient to account for all the urea produced (Lindley et al., 1999). These facts suggest that, in teleosts, the expression of O-UC enzymes in extrahepatic

tissue, particularly muscle, is universal. To date, most investigations related to urea synthesis have been carried out on hepatic tissue; however, it is possible that O-UC enzymes in extrahepatic tissues play an important role in urea synthesis, at least in those teleosts whose urea production is enhanced under stress.

The Gobiidae is the largest family (200 genera) of marine fishes in the world, comprising more than 1875 species (Helfman et al., 1997). They are widely distributed both geographically and ecologically. Their habitat ranges from open sea to small inland streams, and vertically from mud surfaces on land to bottom layers in water. They develop diverse morphological characters and various physiological functions depending on their habitat. The mudskipper *Periophthalmus modestus*, found in Japan, is well adapted to terrestrial life. It has a very high ammonia tolerance, despite a urea production that remains unchanged following exposure to elevated water ammonia levels or to air (Iwata et al., 1981; Iwata, 1988). Further, when the mudskipper was exposed to ^{15}N-labelled ammonia, ^{15}N was heavily incorporated into the amide-N in glutamine, whereas urea-N was relatively unlabelled (Iwata and Deguchi, 1995).

In contrast, in preliminary experiments, we found that the abehaze *Mugilogobius abei*, a small gobiid fish (body mass normally less than 1 g), has both a very high ammonia tolerance and produces a large amount of urea in response to elevated water ammonia levels. Both *M. abei* and *P. modestes* live in mud-flats around the mouth of rivers. In contrast to the mudskipper, the external features of *M. abei* are those of a typical water-breathing gobiid fish. *M. abei* lives under oyster shells and pebbles that remain submerged at ebb tide.

In the mudflats where *M. abei* lives, deoxidized sediments with strongly negative redox potentials are distributed extensively 2–10 cm below the surface. In interstitial water with deoxidized sediments, ammonia concentration is usually very high, and occasionally reaches 2 mmol l^{-1} (K. Iwata, unpublished data). Furthermore, *M. abei* can live in heavily polluted areas (caused by sewage drainage) from which all other fish species have disappeared. It is possible that in some situations, e.g. the water in nest burrows, local ammonia concentrations can be extremely high and the development of a ureogenic ability or other such defence mechanism against ammonia will confer a great benefit to survival. The objectives of the present study were to investigate the ability of *M. abei* to produce urea under ammonia-loading conditions and to establish the pathway for *de novo* synthesis of urea.

We performed the following experiments: (i) urea and ammonia excretion rates were determined under ammonia-loading and control conditions; (ii) the incorporation of ^{15}N-labelled ammonia into urea-N in excreta and into amide-N in glutamine, and urea-N and free amino acid levels in the tissues was measured; (iii) concentrations of ammonia, urea, uric acid and free amino acids in several tissues following exposure to ammonia were compared with those of controls; (iv) CPSase III mRNA levels in various tissues of ammonia-exposed fish were measured using reverse transcription/polymerase chain reaction (RT-PCR); and (v) the activities of O-UC and affiliated enzymes and of uricolytic enzymes were measured.

Materials and methods

Fish collection and holding conditions

Mugilogobius abei (Jordan et Snyder) weighing 0.4–1.0 g were captured with a hand net at the estuary of Wakanoura and Uchinoura in Wakayama Prefecture, Japan. They were kept in 20 % (approximately 7 ‰ salinity) sea water (SW; Jamarin-U synthetic sea water, Jamarin Laboratory, Japan) for 1 week prior to the start of experiments. During this period, a commercial diet (TetraMin, Tetra Werk, Germany) was supplied daily. Throughout the experimental period, the fish were held at 25±1 °C with a constant photoperiod (12 h:12 h L:D). No attempt was made to separate the sexes.

Experimental protocol

Three experiments were performed. (i) Control experiment. After being starved for 2 days, the fish were placed individually in small containers (80 mm diameter) in 20 % SW (synthetic sea water diluted with distilled water, pH 7.6) and kept for 5–9 days during which they were not fed. Water samples (10–15 ml for determination of ammonia and urea excretion) were taken when each fish was transferred to a clean container containing 50 ml of fresh 20 % SW every day. At the same time, blank water samples (20 % SW left for 24 h at 25 °C in the absence of fish) were also taken. The water samples were kept at –20 °C until later analysis. (ii) Exposure to high external ammonia concentrations. After being starved for 2 days, the fish were placed in 20 % SW for 1 day, as in the control experiments, and then in 2 mmol l^{-1} NH_4HCO_3 dissolved in 20 % SW (pH 7.8) for 4–8 days. The external water was changed daily, and both water samples and blank ammonia samples (2 mmol l^{-1} NH_4HCO_3 left for 24 h at 25 °C in the absence of fish) were collected and frozen as for the controls. In addition, similar experiments were conducted using 10 mmol l^{-1} NH_4Cl and 4 mmol l^{-1} NH_4HCO_3 dissolved in 20 % SW. (iii) Incorporation of ^{15}N-labelled ammonia into urea and related nitrogenous substances. The fish were exposed to a ^{15}N-labelled ammonia solution [1 mmol l^{-1} $(^{15}NH_4)_2SO_4$, 99.7 atom % (Shoko Tsusho, Japan) and 2 mmol l^{-1} $NaHCO_3$ dissolved in 10 % SW and adjusted to pH 8.0 with NaOH] for 4 days as in experiment 2. Ambient water samples were taken when the ^{15}N-labelled ammonia solution was renewed every 24 h, and these were kept at –20 °C until analysis.

To measure nitrogenous substances in the tissues, in all three experiments after the respective exposure for 4 days or corresponding control period, 25–30 fish for each experiment was deeply anaethetized by immersion in ice-cold water, blotted with wet gauze and weighed. Liver and muscle tissue samples were removed, and the individual tissue samples and remaining carcass were then weighed and immediately frozen in liquid nitrogen. Frozen tissues were pulverized in a mortar and pestle precooled with liquid nitrogen and then homogenized in cold 80 % ethanol using a sonicator. Because

Table 1. *Effects of antibiotics on measurements of ammonia and urea excreted by fish*

	Ammonia (μmol-N l^{-1})		Urea (μmol-N l^{-1})	
A	Without	With	Without	With
Sea water	52.59±3.68 (12)	54.19±3.19 (12)	17.76±2.02 (12)	19.08±1.81 (12)
NH_4HCO_3	ND	ND	93.96±7.49 (12)	75.61±9.31 (12)
	Ammonia (%)		Urea (%)	
B	Without	With	Without	With
Sea water	101.7±2.8 (12)	108.5±4.5 (12)	101.8±2.8 (12)	99.4±3.3 (12)
NH_4HCO_3	ND	ND	94.6±3.1 (12)	103.9±5.8 (12)

Values are means ± S.E.M. (*N*); ND, not determined.

(A) Each fish was kept for 24 h at 25 °C in a holding container with 20 % sea water or 2 mmol l^{-1} NH_4HCO_3 in the presence (with) or absence (without) of antibiotics, and ammonia and urea concentrations were then measured.

(B) The solutions in which the fish had been kept for the first 24 h were incubated for a further 24 h at 25 °C in the presence or absence of antibiotics, and ammonia and urea concentrations were then measured and expressed as a percentage of the pre-incubation values.

SW, sea water.

of the small sizes of the samples, tissues from two or three fish in each experiment were pooled. Homogenates were kept at –20 °C until analysis.

Microbial effects on excreted urea

Urea excreted by the fish during a 24 h period may be significantly degraded to ammonia through the action of bacteria at the experimental temperature (25 °C). Also, ammonia may be converted to urea by microbial enzymes in the fish containers. To examine bacterial effects, 0.5 ml of antibiotic antimycotic solution (Sigma; 10 000 units ml^{-1} penicillin, 10 mg ml^{-1} streptomycin, 25 mg ml^{-1} amphotericin B in 0.9 % NaCl) was added to each holding container (50 ml of 20 % SW or 2 mmol l^{-1} NH_4HCO_3) in which the fish was kept for 24 h, and the levels of excreted urea and ammonia (in the 20 % SW experiment) in the presence and absence of antibiotics were compared (see below for analytical procedures). Furthermore, the solution (20 % SW or 2 mmol l^{-1} NH_4HCO_3) in which the fish had been kept for the first 24 h was left for a further 24 h at 25 °C in the presence and absence of the antibiotics (the same dosage as above), and ammonia and urea concentrations were compared with those before incubation. As shown in Table 1, the urea concentration measured after 24 h in 2 mmol l^{-1} NH_4HCO_3 in the presence of antibiotics tended to be somewhat lower than that measured in the absence of antibiotics, although there was no significant difference between the two (Student's *t*-test, $P<0.09$). When the solutions in which the fish were kept for the first 24 h were left for a further 24 h, there was no significant change in urea and ammonia concentrations irrespective of the presence or absence of antibiotics. On the basis of these results, all the present experiments on urea and ammonia excretion were performed without adding antibiotics.

Analytical procedures

Water analysis

Ammonia and urea levels in the external water were analyzed using the colorimetric assays described by Solorzano (1969) and Ceriotti and Spandrio (1963), respectively. Ammonia excretion in the presence of 2 mmol l^{-1} NH_4HCO_3 was estimated, after 1:100 dilution of the respective samples with deionized water, from the difference in ammonia concentrations in the presence and absence (blank ammonia sample) of fish.

Determination of levels of ammonia, urea, citrulline, uric acid and free amino acids in tissues

The procedures for extracting and concentrating the water-soluble tissue non-protein fraction (tissue extracts) from the 80 %-ethanol-extracted tissue homogenates (see above) were as described by Iwata and Deguchi (1995). The total ammonia content ($NH_3 + NH_4^+$) in the tissue extracts was determined by ion chromatography (HIC-6A, Shimazu Co., Japan) with a Shim-pack IC-C1 column. Free amino acid contents were measured using a Shimadzu amino acid analysis system (ISC-07/S1504 Li type column). The method for simultaneous determination of urea and citrulline levels in the tissue extracts was similar to that of Blumenkrantz and Asboe-Hansen (1975), except that the chromogen reagent described by Rahmathullah and Boyde (1980) was used. The urea concentration in the tissue extracts was estimated from the difference in chromogenic reaction before and after urease (Type IX, Sigma) digestion: 10 units of urease was added to each filtrate (10–50 μl), which was then incubated for 1 h at 30 °C. After digestion of urea in the tissue extract with urease, citrulline concentration was determined using the same colorimetric method. Uric acid concentration was determined colorimetrically by a modification of the alkaline phosphotungstate method (Henry et al., 1957).

Fractionation of ^{15}N-labelled substances

^{15}N-enrichment in urea excreted into the external water was determined as follows. Because of low levels of urea, the water samples from two fish were pooled. The water samples

(80 ml) was adjusted to pH 10 with NaOH and evaporated *in vacuo* with a rotary evaporator at 50 °C. The dried sample was dissolved in a small amount of distilled water and evaporated again after adding 1 ml of 2 mol l^{-1} NH_4OH to remove any trace of ^{15}N-labelled ammonia. The resulting dried sample was neutralized with 6 mol l^{-1} HCl. After adding 0.2 ml of 0.2 mol l^{-1} phosphate buffer (pH 7.2) and 10 units of urease (Type IX), neutralized samples were incubated at 35 °C for 1 h, and the liberated ammonia was trapped on a fibreglass filter, as described previously (Iwata and Deguchi, 1995).

Procedures for the fractionation of the water-soluble non-protein tissue fraction into ammonia-N, urea-N and amino-N fractions (no attempt was made to separate the amino acid fraction into individual amino acids) were as described by Iwata and Deguchi (1995). The separation of amide-N from the non-protein fraction was modified as follows: after removal of the ammonia-N from the non-protein fraction by adding 0.1 ml of 2 mol l^{-1} Na_2CO_3, the filtrate was dried by bubbling with nitrogen at 50 °C after adding 0.1 ml of 2 mol l^{-1} NH_4OH. The resulting dried sample was neutralized with 1 mol l^{-1} HCl, and 50 µl of 0.1 mol l^{-1} sodium acetate buffer (pH 4.9) and 2 units of glutaminase (Grade II, Sigma) were then added to each filtrate. The ammonia liberated after incubation for 1 h at 35 °C was trapped on a fibreglass filter as described by Iwata and Deguchi (1995).

The isotope ratio ($^{15}N/^{14}N$) in each fraction was measured using a quadrupole-type mass spectrometer equipped with a CN analyzer (Anerva, TE-360B, Japan), and ^{15}N-enrichment in each fraction was expressed as atom % excess (atom % of each sample minus ^{15}N natural abundance). Atom % of control samples was 0.385±0.027 % (mean ± S.E.M., N=20), i.e. not significantly different from the natural abundance of ^{15}N (Student's t-test, t=0.61).

Sequencing of CPSase-III-specific cDNA segments

Freshly excised tissues from *M. abei* were immediately frozen in liquid nitrogen and then stored at –80 °C. Poly(A^+) RNA was obtained from approximately 0.1 g of pooled muscle by oligo(dT) Sepharose chromatography using a Microprep RNA kit (Pharmacia). Poly(A^+) RNA (100 ng) was used to synthesize the first-strand cDNA using the first-strand cDNA synthesis kit (Pharmacia). The instructions for the kit were followed using oligo$(dT)_{18}$ primer (33 µl reaction volume). After heating at 90 °C for 5 min, the samples were stored at –20 °C.

Primers, 5′-TGGAARGARGTIGARTAYGA-3′ (sense) and 5′-GTYTCNGGRTTRTGRTTNACIAC-3′ (antisense), with 5 µl of cDNA in a 50 µl reaction mixture were used for the first polymerase chain reaction (PCR) (KOD Dash, Toyobo, Japan). The DNA thermal cycler was programmed for touchdown PCR (Korte et al., 1997): the first cycle was 5 min at 94 °C, 1 min at 55 °C and 1 min at 74 °C; the next two cycles were 1 min at 94 °C, 1 min at 54 °C and 1 min at 74 °C. In subsequent cycles, the annealing temperature was decreased using this pattern until the annealing temperature was 50 °C, and the cycle was then repeated a total of 30 times. This stage of the PCR was repeated using 1 µl of product from the initial PCR using the same primers and programs.

The second stage of the PCR was then carried out with the primer 5′-ARAARACNACNGCNTG-3′ (sense) and the antisense primer listed above using 1 µl of PCR product from the first-stage amplification in a 50 µl standard reaction mixture (Ex Taq, Takara, Japan). The thermal cycler was programmed as follows: 94 °C for 1 min, 48 °C for 1 min and 72 °C for 2 min; this cycle was repeated for a total of 40 cycles. This procedure resulted in several products when analyzed by gel electrophoresis on 2 % agarose gel in TBE buffer (89 mmol l^{-1} trishydroxymethyl aminomethane, 89 mmol l^{-1} boric acid and 2 mmol l^{-1} EDTA, pH 8.0) (detected by ethidium bromide staining).

A nested reamplification of the product from the second stage (1 µl) was carried out with the primers 5′-GTNGG-NGARGTNATGGCNG-3′ (sense) and 5′-NCCDATRTGR-TANGG-3′ (antisense). The PCR conditions used for reamplification were: 95 °C for 1 min, 51 °C for 1 min and 75 °C for 2 min; this cycle was repeated for a total of 35 cycles. A major band at approximately 579 base pairs (bp), which corresponds in size to amino acid residues 796–988 of largemouth bass CPSase III (Kong et al., 1998), was observed following electrophoresis. This product was cut from the gel, extracted and cloned in pT7Blue vector (Novagen, Madison, WI, USA) prior to sequencing. The sequence of both cDNA strands was determined using a *Taq* dye terminator cycle sequencing kit (Perkin-Elmer, Foster City, CA, USA).

RT-PCR assay for CPSase III expression

Using reverse transcription/polymerase chain reaction (RT-PCR), we assayed for the presence of CPSase III mRNA in various tissues, including liver, intestine, spleen, muscle, skin and gill of fish exposed to 2 mmol l^{-1} NH_4HCO_3 for 5 days. Tissue samples from two or three fish were pooled. The primers 5′-AAGGCCCTGAGGATGTGTCA-3′ (sense) and 5′-TGCTTCTCCAGCTGTGT-3′ (antisense) were designed to give CPSase-III-specific RT-PCR products which included the gap area of CPSase II (see Fig. 3). Poly(A^+) RNA was obtained from approximately 0.1 g of pooled tissue by oligo(dT) Sepharose chromatography as described above. Poly(A^+) RNA (100 ng) was used to synthesize the first-strand cDNA using the first-strand cDNA synthesis kit (Pharmacia) and oligo$(dT)_{18}$ primer in a volume of 33 µl. PCR amplification was performed using 0.5 µl of the RT reaction and Ex Taq (30 cycles of 95 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min), and size-fractionated on a 1 % agarose gel. A product of 239 bp was expected. CPSase III mRNA expression was detected in muscle, skin and gills. Controls had no reverse transcriptase in the reaction to verify that the RT-PCR products of CPSase III were amplified from mRNA, not from genomic DNA. Southern blot analysis of the PCR products using a [α-^{32}P]-labelled oligonucleotide probe, 5′-AGAGGCAGC-AGCCGGGGCATGAATCCATCCACAGAGGGGTGAC-3′, corresponding to the internal sequence unique to the CPSase III

(see Fig. 3), confirmed the identity of the amplified fragment (data not shown).

Quantification of differential CPSase III expression levels

Total RNA was extracted using an RNA extraction kit (Isogen, Nippon Gene, Tokyo, Japan) and quantified spectrophotometrically. Reverse transcriptase reactions were carried out using an oligo(dT)$_{18}$ primer, the first-strand cDNA synthesis kit and 5 μg of total RNA in a volume of 33 μl. After the RT reaction, samples were used as a template for PCR amplification with CPSase-III-specific primers, as described above. As an internal control for the integrity of the mRNA in each sample, additional PCR amplifications were performed using primers designed on the basis of conserved sequences of several glyceraldehyde-3-phosphate dehydrogenases (GAPDHs) that produce a product of 934 bp. The reaction products were separated by agarose gel electrophoresis (2 %), stained with ethidium bromide and quantified by ultraviolet transillumination. Images were captured from video using a CCD monochrome video camera module (Sony) and NIH Image software (NIH).

The RT-PCR assay was validated by determining the linear range of amplification of both CPSase III and GAPDH from 15–40 thermocycles with varying amounts of cDNA from tissues with the highest CPSase III expression levels (ammonia-exposed fish) to determine the upper limits of the linear range of the detection. Between 20 and 40 amplification cycles gave an approximately linear range; the use of 30 cycles allows substantial signal detection of low-level tissue expression and is at the upper limit for measurement of high expression levels. After 30 cycles of amplification, the linear range of detection of both mRNAs extended from 0.1 to 0.5 μl of cDNA produced by RT. The use of 0.1–0.3 μl of cDNA in the PCR reactions as a template also allowed for measurement of expression over the linear range of amplification, even in the highest-expressing tissues (data not shown). Values were normalized to the quantity of GAPDH signal and quantified for CPSase III expression using arbitrary units.

Enzyme analysis

After exposure to 2 mmol l^{-1} NH_4HCO_3 for 4–5 days, the fish were deeply anaesthetized by immersion in ice-cold water, and the liver, muscle, skin and gill tissue were then removed. Because of the small sizes of the samples, tissues from 3–5 fish were pooled. Freshly excised tissues were either used immediately (e.g. for CPSase activity) or stored at −80 °C until needed. Tissues obtained from fish starved for 4–5 days in 20 % SW served as controls.

Tissues were minced and homogenized (Ultra-Turrax T25 homogenizer) in 10 volumes of the extraction buffer described by Korte et al. (1997). The homogenate was subjected to brief sonication and then centrifuged at 15 000 ***g*** for 20 min at 4 °C; the supernatant was used directly for enzyme assays (except for CPSase). To assay CPSase, approximately 0.6 ml of the above supernatant was passed through a 1 cm×6 cm (approximately 5 ml) column of Sephadex G-25 equilibrated with extraction buffer; the majority of protein was collected in approximately 1.0 ml of eluant, and this was used immediately in the enzyme assay. The protein concentration of the extracts was measured before and after the gel filtration chromatography step to adjust for dilution when calculating units in g tissue^{-1}. All enzyme activities were assayed at 30 °C and are expressed per gram of wet tissue (μmol min^{-1} g^{-1} or nmol min^{-1} g^{-1}).

Carbamoyl phosphate synthetase (CPSase) activity was assayed by a micro-modification of the procedure described by Korte et al. (1997). The standard reaction mixture contained 20 mmol l^{-1} ATP, 25 mmol l^{-1} $MgCl_2$, 25 mmol l^{-1} phosphoenolpyruvate, 2 units of pyruvate kinase, 5 mmol l^{-1} $NaH^{14}CO_3$ (2×10^6 counts min^{-1}), 20 mmol l^{-1} glutamine, 2 mmol l^{-1} *N*-acetylglutamic acid (AGA), 2 mmol l^{-1} uridine triphosphate (UTP), 0.04 mol l^{-1} Hepes, pH 7.6, 0.04 mol l^{-1} KCl, 0.5 mmol l^{-1} EDTA, 0.5 mmol l^{-1} dithiothreitol and extract (50–150 μl) in a final volume of 0.3 ml. Carbamoyl phosphate formed after 60 min of incubation was determined as described by Korte et al. (1997). In addition to this CPSase assay with glutamine as the nitrogen-donating substrate, CPSase activity was also assayed with ammonia as the substrate: 5 mmol l^{-1} NH_4HCO_3, 10 mmol l^{-1} ornithine and 2 units of ornithine carbamyl transferase (Sigma; purified from *Streptococcus faecalis*) were added to the above reaction mixture (in this case $NaH^{14}CO_3$ and glutamine were omitted), and the citrulline formed after incubation was measured spectrophotometrically by a modification of the procedure described by Pierson (1980).

The reaction mixture for ornithine transcarbamoylase (OTCase) assay was that described by Wright et al. (1995). After a 15 min incubation, the reaction was terminated by adding 0.5 mol l^{-1} $HClO_4$ (PCA), and the citrulline formed was determined colorimetrically (Pierson, 1980).

Argininosuccinate synthetase and argininosuccinate lyase (ASSase/ASLase) were assayed by a modification of the method of Brown and Cohen (1959). The reaction mixture consisted of 5 mmol l^{-1} L-aspartic acid, 5 mmol l^{-1} $MgCl_2$, 5 mmol l^{-1} L-citrulline, 10 mmol l^{-1} ATP, 80 mmol l^{-1} phosphate buffer (pH 7.0), 20 units of arginase (Sigma) and the enzyme extract (50–100 μl) in a final volume of 0.5 ml. After incubation for 60 min, the reaction was terminated by adding 0.1 ml of 0.5 mol l^{-1} PCA. The PCA-treated system served as the control. A reduction in citrulline concentration after urease digestion [after neutralization with KOH, 20 units of urease (Type IX) was added and the mixture was incubated for 30 min at 30 °C] was determined colorimetrically as for the OTCase assay.

Arginase (ARGase) activity was measured as described by Campbell et al. (1968). Glutamine synthetase (GSase) activity, assayed *via* the formation of γ-glutamyl-hydroxamate, was measured by a micro-modification of the method of Webb and Brown (1976). Uricase and allantoicase activities were determined by the method of Brown et al. (1966). Glutamate dehydrogenase (GDH) activity was assayed in the direction of reductive amination as described previously (Iwata et al., 1981).

Statistical analyses

Data are presented as means ± S.E.M. (N). The value for the whole body is the sum of the tissue values times the mass of each tissue sample. The significance of differences between means (P<0.05) was tested using Student's t-tests or two-way analysis of variance (ANOVA).

Results

Ammonia and urea excretion

The rates of ammonia and urea excretion of the control fish were relatively stable over time (Fig. 1) and showed no significant variation with duration of starvation. The mean (N=14) rates of ammonia and urea excretion throughout the experimental period (hereafter referred to as the control mean ammonia or urea excretion rate) were 13.16± 0.53 and 2.31±0.24 μmol-N day^{-1} g^{-1} wet mass, respectively, with the percentage of nitrogen excreted as urea accounting for 14.9±0.4 % (N=14) of total nitrogen excretion (ammonia-N + urea-N).

In 2 mmol l^{-1} ammonium bicarbonate or 10 mmol l^{-1} ammonium chloride, *M. abei* survived indefinitely without any adverse effects. However, when exposed to 4 mmol l^{-1} ammonium bicarbonate, the majority of fish died within 24 h.

The rates of urea excretion of the fish exposed to 2 mmol l^{-1} ammonium bicarbonate increased over time, levelling off by day 4 (Fig. 1A). Thereafter, urea excretion remained approximately seven times higher than the control value. There were no significant differences among the rates of urea excretion from days 4–8 after exposure; the mean of these rates (13.72±0.85 μmol-N day^{-1} g^{-1} wet mass, N=10) was not significantly different from the control mean total nitrogen excretion. Similar results were obtained in the fish exposed to 10 mmol l^{-1} ammonium chloride dissolved in 20 % SW (data not shown).

On the first day of 2 mmol l^{-1} ammonium bicarbonate exposure, the rate of ammonia excretion was reduced to one-eighth of the control rate. Between days 2 and 4, the rates gradually increased but remained significantly lower than the control mean ammonia excretion (Fig. 1B). Between days 5 and 7, rates were not significantly different from those of the corresponding control fish. However, the mean ammonia excretion rate (8.52±0.91 μmol-N day^{-1} g^{-1} wet mass, N=10) for days 5–8 following exposure was significantly lower than the control mean ammonia excretion rate, suggesting that ammonia excretion was still inhibited after 4 days of exposure. Using the mean rates of ammonia and urea excretion during the period between days 4 and 8, the percentage of nitrogen excreted as urea accounted for 61.7 % of total nitrogen excretion.

Incorporation of [^{15}N]ammonia into urea-N, amide-N and amino-N

Following exposure to ^{15}N-labelled ammonia solution, increases in urea excretion rate with time were similar to those in 2 mmol l^{-1} ammonium bicarbonate (no significant difference between the two treatments) (compare Fig. 2 with Fig. 1A). ^{15}N-enrichment in excreted urea-N increased over time,

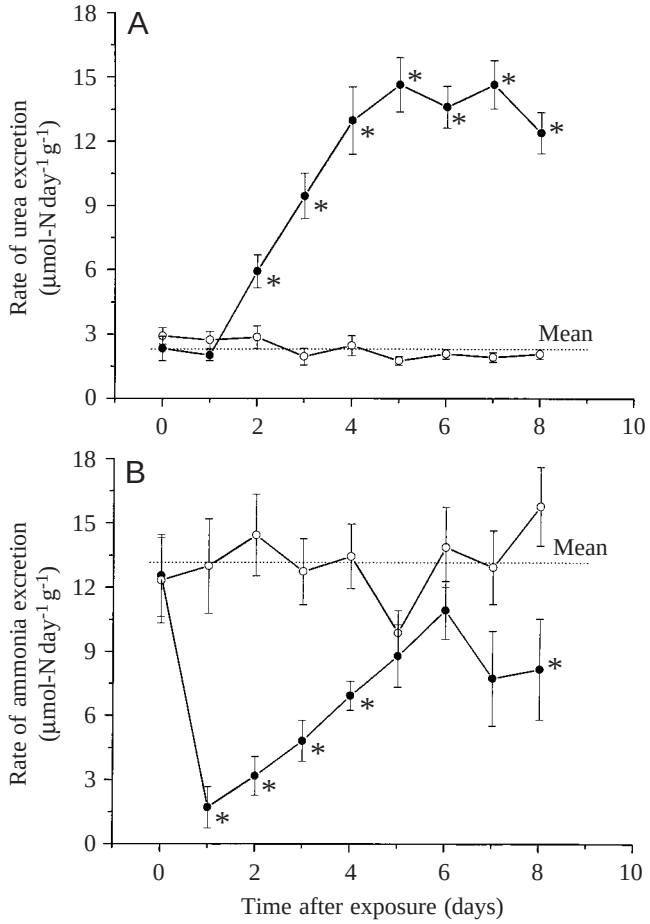

Fig. 1. Time course of changes in (A) urea and (B) ammonia excretion rates in starved *Mugilogobius abei* exposed to 2 mmol l^{-1} ammonium bicarbonate (filled circles, N=10) and in the 20 % seawater control fish (open circles, N=14). Values are means ± S.E.M. An asterisk indicates a significant difference (P<0.05) from the corresponding starved control value. The dotted line represents the mean urea or ammonia excretion rate of the control fish.

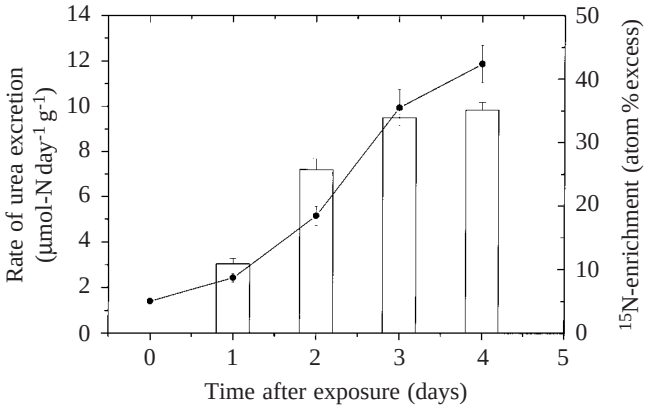

Fig. 2. Time course of changes in urea excretion rates (filled circles, N=10) and ^{15}N-enrichment (atom % excess) of excreted urea-N (columns) in starved *Mugilogobius abei* exposed to ^{15}N-labelled ammonia solution (N=10). Means ± S.E.M. are plotted for each day.

Table 2. *^{15}N-enrichment (atom % excess) of the ammonia-N, amide-N in glutamine, urea-N and amino-N fractions in the tissues and excreta following exposure to ^{15}N-labelled ammonia solution for 4 days*

	Liver	*N*	Muscle	*N*	Whole body	*N*	Excreta	*N*
Ammonia-N	62.31±2.92	4	61.06±1.15	6	62.42±1.55	6	ND	
Urea-N	35.51±0.99	4	32.19±1.06	6	33.19±1.67	6	35.95±1.12	10
Amide-N	64.54±1.68	4	60.31±1.99	6	58.25±1.44	6		
Amino-N*	3.22±0.38	4	1.51±0.07	6	2.28±0.16	6		

Values are means ± S.E.M.; ND, not determined.
An asterisk indicates that the ^{15}N-enrichment level was significantly different between tissues.

reaching a plateau at day 3 (no significant difference between days 3 and 4), indicating that the nitrogen pool relating to urea production was equilibrated with ^{15}N within 3 days following exposure to ^{15}N-labelled ammonia.

^{15}N-enrichment in urea-N was similar in the different tissues (no significant differences), and these values were also not significantly different from that in the excreta (Table 2). ^{15}N-enrichment in ammonia-N and amide-N in glutamine were also not significantly different among the tissues examined, but were approximately twice the urea-N values. ^{15}N-enrichment in the amino-N fractions was very low compared with that in amide-N, but was significantly different among tissues (liver>whole body>muscle).

Ammonia, urea, uric acid and free amino acid contents

As shown in Table 3, ammonia contents in the tissues of ammonia-exposed fish were 2–3 times those of controls. Urea levels in the muscle and whole body, but not in the liver, also increased approximately threefold following ammonia exposure, but uric acid levels remained unchanged in all the tissues examined.

Of the free amino acids measured, glutamine content in all the tissues examined increased greatly following exposure and reached levels 4–6 times that of the control (P<0.0001). Alanine concentrations in the muscle and whole body of ammonia-exposed fish were also significantly higher than that of the control (P<0.01). Proline levels decreased significantly (P<0.05) in the whole body following exposure. It should be noted that citrulline levels in the muscle and whole body of the exposed fish increased to approximately twice those of controls (P<0.05 and P<0.01, respectively).

Sequence of CPSase III cDNA from M. abei

The alignments of known teleost CPSase II and III

Table 3. *Changes in free amino acid content and ammonia, urea, uric acid and citrulline contents (μmol N g^{-1} tissue) in liver, muscle and whole body following exposure to ammonia for 4 days*

	Liver				Muscle				Whole body			
	Control		Ammonia-exposed		Control		Ammonia-exposed		Control		Ammonia-exposed	
	(μmol g^{-1})	*N*	(μmol g^{-1})	*N*	(μmol g^{-1})	*N*	(μmol g^{-1})	*N*	(μmol g^{-1})	*N*	(μmol g^{-1})	*N*
Tau	7.74±0.94	10	7.01±0.64	10	12.6±1.11	10	9.81±0.82	10	8.95±0.94	10	7.20±0.25	10
Asp	0.33±0.05	10	0.25±0.02	10	0.37±0.08	10	0.23±0.06	10	0.42±0.06	10	0.29±0.07	10
Thr	0.55±0.06	10	0.34±0.03	10	1.14±0.13	10	0.94±0.18	10	0.74±0.16	10	0.61±0.07	10
Ser	0.51±0.06	10	0.34±0.04	10	0.77±0.10	10	0.74±0.07	10	0.55±0.08	10	0.46±0.04	10
Asn	0.23±0.04	10	0.18±0.05	10	0.87±0.14	10	1.30±0.15	10	0.65±0.22	10	0.93±0.17	10
Glu	2.89±0.63	10	1.97±0.24	10	0.58±0.06	10	0.68±0.14	10	1.38±0.14	10	1.18±0.11	10
Gln	1.73±0.59	10	5.60±1.22	10***	1.12±0.20	10	6.86±1.53	10***	0.83±0.16	10	5.30±0.73	10***
Pro	0.59±0.16	10	0.33±0.12	10	0.61±0.13	10	0.37±0.11	10	0.35±0.05	10	0.19±0.02	10*
Gly	0.89±0.26	10	0.58±0.18	10	9.39±1.38	10	8.74±0.73	10	3.95±1.06	10	4.89±0.58	10
Ala	0.97±0.28	10	0.81±0.11	10	0.87±0.21	10	1.89±0.29	10**	0.63±0.16	10	1.31±0.13	10**
	(μmol-N g^{-1})	*N*	(μmol-N g^{-1})	*N*	(μmol-N g^{-1})	*N*	(μmol-N g^{-1})	*N*	(μmol-N g^{-1})	*N*	(μmol-N g^{-1})	*N*
Amm	3.59±0.40	6	7.83±1.00	6**	4.98±1.04	10	13.95±1.16	10***	3.92±0.41	10	11.43±0.33	10***
Urea	3.51±0.84	6	2.04±0.42	6	2.85±1.31	10	6.81±1.35	10***	1.62±0.32	10	5.51±1.10	10***
Cit	0.21±0.06	6	0.33±0.03	6	0.36±0.08	10	0.69±0.12	10*	0.25±0.04	18	0.57±0.06	18**
Uric acid	ND		ND		0.29±0.05	6	0.35±0.10	6	0.47±0.09	17	0.67±0.12	16

Values are means ± S.E.M.; ND, not determined.
Tau, taurine; Asp, aspartate; Thr, threonine; Ser, serine; Asn, asparagine; Glu, glutamate; Gln, glutamine; Pro, proline; Gly, glycine; Ala, alanine; Amm, ammonia; Cit, citrulline.
Asterisks indicate a significant difference from the control value: *P<0.05, **P<0.01 and ***P<0.001.

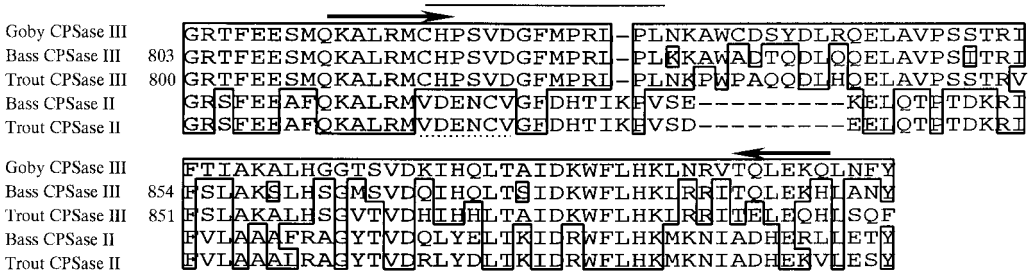

Fig. 3. Alignment of the partially deduced amino acid sequence of carbamoyl phosphate synthetase (CPSase III) of *Mugilogobius abei* (goby) compared with those of the rainbow trout and largemouth bass and with CPSase II of the trout and bass. Residues matching goby CPSase III are indicated by boxes. Gap regions are indicated by dashes. The dotted line marks a putative conserved sequence unique to CPSase II. The location of the primer used for quantitative reverse transcription/polymerase chain reaction (RT-PCR) and the probe used for Southern blot analysis are indicated by arrows and the solid line, respectively.

sequences are shown in Fig. 3 with the derived amino acid sequence of CPSase III of *M. abei*. This partial sequence was identified as a CPSase III cDNA fragment on the basis of the following evidence. (i) This sequence displayed approximately 40 % amino acid identity to CPSase II from largemouth bass (Kong et al., 1998) and rainbow trout (Korte et al., 1997), whereas the optimized alignment of the corresponding regions of CPSase III obtained from largemouth bass and rainbow trout revealed an approximately 80 % identity among the sequences (Clustal method). (ii) The sequence VDENCV, unique to CPSase II, was replaced by the sequence of the corresponding region of bass and trout CPSase III. (iii) The sequence did not have a gap region, a feature unique to CPSase II. The partial nucleotide and derived amino acid sequence of *M. abei* CPSase III is available from GenBank under Accession No. AB040100.

Expression of CPSase III mRNA in various tissues

RT-PCR of RNA isolated from various tissues of *M. abei* was used to look for the presence of CPSase III mRNA. As shown in Fig. 4, CPSase III mRNA was detected in the skin, muscle and gills, but not in the liver, spleen and intestine. Fig. 5 shows the results of a semi-quantitative analysis of CPSase III mRNA expression in the skin, muscle and gill. CPSase III expression was measured using a RT-PCR-based assay in which the internal standard GAPDH was used to estimate equivalent amounts of mRNA pools (there was no apparent difference in GAPDH mRNA expression between the exposed and control fish). The RT-PCR amplification products were quantified by densitometry and expressed as relative values, the ratio of CPSase III expression to that of GAPDH.

Relative CPSase III mRNA expression in the skin of the ammonia-exposed fish was not different from that of controls. Relative CPSase III mRNA expression in muscle increased twofold following ammonia exposure (P<0.07). Relative CPSase III mRNA expression in the gill increased threefold (P<0.01) in response to ammonia exposure.

Activities of O-UC enzymes

In spite of the significant increase in relative CPSase III mRNA expression in gill following ammonia exposure, the

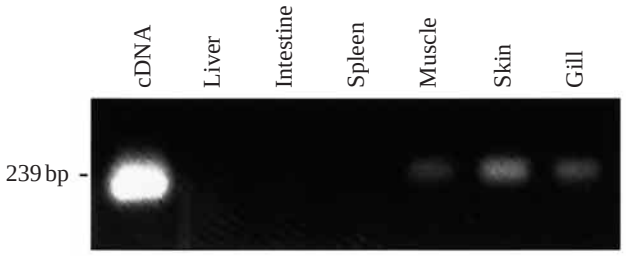

Fig. 4. Carbamoyl phosphate synthetase III (CPSase III) gene expression in various tissues of *Mugilogobius abei*. CPSase III mRNA expression was detected by the reverse transcription/polymerase chain reaction (RT-PCR) method as described in Materials and methods.

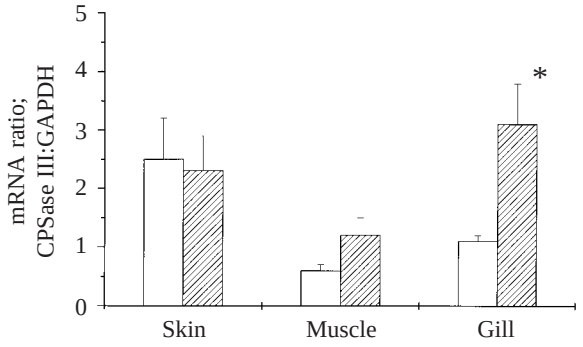

Fig. 5. Semi-quantitative reverse transcription/polymerase chain reaction (RT-PCR) analysis of carbamoyl phosphate synthetase III (CPSase III) mRNA expression. CPSase III mRNA expression was measured by RT-PCR using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as an internal standard. The RT-PCR amplification products were quantified by densitometry and are expressed as a relative value, the ratio of CPSase III mRNA to GAPDH mRNA expression. The open and hatched columns indicate relative expression in the control and ammonia-exposed fish, respectively. Values are means ± S.E.M. N=6 except for control gill (N=5). An asterisk indicates a significant difference (P<0.01) from the control value.

Table 4. *Activities of enzymes related to the ornithine–urea cycle in liver, muscle, skin and gill following exposure to ammonia for 4–5 days*

	Liver		Muscle		Skin		Gill	
CPSase	(nmol min^{-1} g^{-1})	*N*	(nmol min^{-1} g^{-1})	*N*	(nmol min^{-1} g^{-1})	*N*	(nmol min^{-1} g^{-1})	*N*
Gln+UTP	0.04±0.013	6	1.40±0.39	6	2.03±0.46	5	1.17±0.28	4
Gln+AGA+UTP	0.09±0.015	6	2.69±0.69	6	2.87±0.40	5	1.94±0.22	4
Amm	ND		0	4	0	4	ND	
Amm+AGA	ND		1.9±0.4	4	2.7±0.5	4	ND	
Amm+AGA+UTP	ND		1.5±0.3	4	2.2±0.5	4	ND	
	(μmol min^{-1} g^{-1})	*N*	(μmol min^{-1} g^{-1})	*N*	(μmol min^{-1} g^{-1})	*N*	(μmol min^{-1} g^{-1})	*N*
OTCase	0.13±0.02	8	0.63±0.13	8	3.13±1.12	6	3.29±1.21	4
ASSase+ASLase	0.04±0.01	4	0.12±0.03	6	0.09±0	4	0.26±0.04	4
ARGase	7.16±1.07	8	0.03±0.01	8	0.40±0.30	8	2.71±0.54	6

Values are means ± S.E.M.; ND, not determined.

Control values are not shown, but there were no significant differences between controls and ammonia-exposed fish.

CPSase, carbamoyl phosphate synthetase; Gln, glutamine; UTP, uridine triphosphate; AGA, *N*-acetyl-glutamic acid; Amm, ammonia; OTCase, ornithine transcarbamoylase; ASSase, argininosuccinate synthetase; ASLase, argininosuccinate lyase; ARGase, arginase.

CPSase activity was assayed with glutamine or with ammonia as a substrate, see Materials and methods.

activities of CPSase as well as other O-UC enzymes were not significantly different between treatments, so only the enzyme activities in tissues of the ammonia-exposed fish are shown in Table 4.

In muscle, skin and gill, CPSase activities with glutamine as the nitrogen-donating substrate tended to increase in the presence of AGA (92, 44 and 66 % for muscle, skin and gill, respectively), although these levels in the presence of AGA were not statistically different from those in the absence of AGA. The levels in liver were very low irrespective of the presence or absence of AGA. CPSase activities in muscle and skin with ammonia as the substrate were similar to those with glutamine as the substrate, but CPSase required the presence of AGA to be activated. For both substrates, UTP had little effect on enzyme activity. Taken together, this suggests that CPSase activities detected in the muscle, skin and gill are primarily due to CPSase III, although these properties are considerably different from those of other CPSase IIIs (Anderson, 1995).

OTCase activities in skin and gill were 5 and 30 times higher than those of muscle and liver, respectively. Similarly, ASSase/ASLase activities in muscle, skin and gill were 3–6 times higher than in liver. Arginase activity in liver was the highest among the tissues examined. Thus, all O-UC enzymes were present and/or functional in the muscle, skin and gill, but probably not in the liver.

Activities of GDH, GSase, uricase and allantoicase

In accord with an elevation of glutamine levels in tissues (see Table 3), GSase activities in liver and muscle of the exposed fish increased to approximately 20 and five times those of controls, respectively, but there was no significant difference in GSase activities in skin and gill (Table 5). GDH activity decreased in the order liver, gill, skin and muscle, and there were no significant differences between treatments. The highest ^{15}N-enrichment of the amino-N fraction was observed in the liver (Table 2). This may be correlated with the observed higher level of GDH in this organ. In the purine degradation pathway, uricase and allantoicase activities in the liver and muscle were not significantly different from those of the controls.

Discussion

The first notable finding of the present study is that ammonia-exposed *M. abei* produced a large amount of urea, representing 62 % of the total nitrogen excreted, and sustained that level over 5 days. Furthermore, ^{15}N-labelled ammonia that entered the fish body was efficiently converted into urea-N (i.e. *de novo* synthesis of urea occurred), indicating that *M. abei* demonstrates functional ureogenesis or facultative ureotely. In teleosts, the two known pathways for *de novo* synthesis of urea are *de novo* synthesis of purine and its subsequent degradation (uricolysis pathway) and the O-UC (Wood, 1993; Anderson, 1995; Mommsen and Walsh, 1991). As shown in Table 2, ^{15}N-enrichment of urea-N was approximately half that of ammonia-N or amide-N in glutamine. Since half the urea-N arises from the amide-N in glutamine and half from aspartate or glycine (if urea is produced *via* the uricolysis pathway), the observed ^{15}N-enrichment in urea could occur *via* either synthetic pathway if ^{15}N-labelled ammonia is mainly incorporated into the amide-N in glutamine but little into α-amino-N in individual amino acids. In fact, in mammalian liver and brain, the amide-N in glutamine is known to be heavily labelled after infusion of ^{15}N-labelled ammonia, but there is little labelling of α-amino-N in glutamine and glutamate (Takagaki et al., 1961; Berl et al., 1962).

In mammals, it is well known that an exogenous ammonia

Table 5. *Effects of ammonia exposure on activities of GSase, GDH and uricolysis enzymes in various tissues of* Mugilogobius abei

	Liver				Muscle			
	Control		Ammonia-exposed		Control		Ammonia-exposed	
	(μmol min^{-1} g^{-1})	*N*	(μmol min^{-1} g^{-1})	*N*	(μmol min^{-1} g^{-1})	*N*	(μmol min^{-1} g^{-1})	*N*
GDH	13.37±1.44	7	18.99±2.61	6	0.58±0.07	7	0.64±0.10	6
GSase	0.28±0.04	8	6.08±0.98	8***	0.10±0.01	8	0.45±0.09	8***
Uricase	0.55±0.06	8	0.64±0.12	8	0.01±0.01	8	0.01±0	8
Allantoicase	0.28±0.08	7	0.53±0.17	8	0	8	0	8

	Skin				Gill			
	Control		Ammonia-exposed		Control		Ammonia-exposed	
	(μmol min^{-1} g^{-1})	*N*	(μmol min^{-1} g^{-1})	*N*	(μmol min^{-1} g^{-1})	*N*	(μmol min^{-1} g^{-1})	*N*
GDH	0.81±0.07	7	0.62±0.11	7	2.35±0.62	4	2.39±0.22	4
GSase	0.15±0.02	5	0.15±0.02	5	0.81±0.21	4	0.96±0.12	4
Uricase	ND		ND		ND		ND	
Allantoicase	ND		ND		ND		ND	

Values are means ± S.E.M.; ND, not determined.
*** indicates a significant difference from controls (P<0.002).
GDH, glutamate dehydrogenase; GSase, glutamine synthetase.
Ammonia exposure involved treatment with 2 mmol l^{-1} NH_4HCO_3.

load is mostly taken up by muscle and synthesized into glutamine, which is the most important vehicle for intertissue nitrogen transport (Ruderman and Lund, 1972; Ganda and Ruderman, 1976). In teleosts, however, except for a few species such as goldfish *Carassius auratus* (Van Waarde and Kesbeke, 1982) and mudskipper *Periophthalmus modestus* (Iwata, 1988; Iwata and Deguchi, 1995), the role of muscle in glutamine formation is generally considered to be of minor importance (Walton and Cowey, 1977; Chamberlin et al., 1991). In ammonia-exposed *M. abei*, the ammonia, urea, glutamine and alanine contents in tissues underwent the greatest changes (Table 3). The net storage of ammonia, urea, glutamine and alanine in the whole fish body is 7.5, 3.9, 4.5 and 0.7 μmol-N g^{-1}, respectively (Table 3), which approximately represents the net nitrogen storage in the fish body during the first 4 days of exposure to ammonia. Since glutamine has two nitrogen atoms, glutamine-N storage is equivalent to 9 μmol-N g^{-1} if the reductive amination of α-ketoglutarate catalyzed by GDH proceeds in parallel with the amidation of glutamate by GSase; thus, net nitrogen storage is 21.1 μmol-N g^{-1}. In the present study, the following estimation of the nitrogen budget of ammonia-exposed fish can be derived for the first 4 days of ammonia exposure: total ammonia loading for 4 days (60.1 μmol-N g^{-1}) = $\sum$net urea production (excretion rate of the exposed fish minus control rate) for 4 days (22.4 μmol-N g^{-1} from Fig. 1A) + $\sum$ammonia excretion for 4 days (16.6 μmol-N g^{-1} from Fig. 1B) + net nitrogen storage (21.1 μmol-N g^{-1}). If half the urea-N is derived from the amide-N in glutamine, glutamine production for 4 days after exposure would account for 17.7 μmol g^{-1}, which corresponds to 4.4 μmol day^{-1} g^{-1} (0.2 μmol h^{-1} g^{-1}).

In *M. abei* following exposure to ammonia, GSase (γ-glutamyl-transferase activity) activities in muscle and liver were increased five- and 20-fold, respectively (Table 5). These GSase activities of *M. abei* are comparable with those of the Lake Magadi tilapia (Lindley et al., 1999). Since GSase biosynthetic activity is approximately 5 % of the transferase activity (Shankar and Anderson, 1985; Anderson and Walsh, 1995), its estimated biosynthetic activity in the muscle is 0.023 μmol min^{-1} g^{-1} and in the liver is 0.304 μmol min^{-1} g^{-1}, corresponding to 0.69 and 0.37 μmol h^{-1} g^{-1} in the whole fish, if muscle and liver make up 50 % and 2 % of body mass, respectively. The contribution of muscle to glutamine synthesis in the fish body therefore appears to be larger than that of liver, and the GSase biosynthetic rate in muscle alone is sufficient to support the *in vivo* glutamine synthetic rate estimated from the nitrogen budget. Furthermore, the amide-N in glutamine in muscle of fish exposed to ^{15}N-labelled ammonia has virtually the same ^{15}N-enrichment as in ammonia-N, and the glutamine and ammonia contents in muscle of the exposed fish account for 65 and 61 % of those in the whole fish body, respectively (if muscle makes up 50 % of body mass). These facts strongly suggest that the conversion of ammonia into the amide-N in glutamine, i.e. the first step of ammonia detoxification, occurs mainly in muscle. Thus, in *M. abei*, muscle appears to play a pivotal role not only in converting a large amount of ammonia into the amide-N in glutamine, but also in feeding this nitrogen into the O-UC as the primary substrate for CPSase III.

Recently, muscle and other extrahepatic tissues in adult teleosts have been reported to express the urea-cycle-related CPSase III (Korte et al., 1997; Kong et al., 1998; Felskie et al., 1998; Saha et al., 1999; Terjesen et al., 2000). The muscle of

the Lake Magadi tilapia has a very high activity of CPSase III and all other urea cycle enzymes, which may account for the observed high rate of urea production in this species (Lindley et al., 1999). In *M. abei*, CPSase III mRNA expression and all urea cycle enzymes were detected not only in muscle, but also in skin and gill, whereas expression in liver was undetectable. To our knowledge, this is the first report of the presence of all urea cycle enzymes in skin and gill. CPSase III activities in the muscle, skin and gill are approximately 10 times higher than those in the muscle and intestine of rainbow trout, carp and bowfin (Korte et al., 1997; Felskie et al., 1999), but are approximately half, one-quarter and one-sixtieth of those in the muscle of the gulf toadfish *Opsanus beta* (Julsrud et al., 1998), the Indian catfish *Clarias batrachus* (Saha et al., 1999) and the Lake Magadi tilapia (Lindley et al., 1999), respectively.

In *M. abei*, net urea production following 4–8 days of ammonia exposure is approximately 11 μmol urea-N day^{-1} g^{-1} (Fig. 1A), which corresponds to approximately 0.23 μmol urea h^{-1} g^{-1}. It should be noted that this *in vivo* rate of urea synthesis is very close to the *in vivo* rate of glutamine synthesis estimated from the nitrogen budget during the first 4 days after exposure. Assuming that muscle and skin together contain 2.7 nmol min^{-1} g^{-1} of CPSase III activity (Table 4) and make up approximately 50 and 15 % of body mass, respectively, the muscle and skin could give rise to approximately 0.11 μmol urea h^{-1} g^{-1}, or approximately half the observed rate of urea production. Given the very low CPSase III activity and undetectable levels of CPSase III mRNA expression in the liver and the small proportion of total tissue mass that it represents (approximately 2 % of body mass), the role of the liver of *M. abei* in urea production *via* the O-UC will be negligible. In contrast, the extra-hepatic tissues, muscle and skin appear to be primarily responsible for urea production, although CPSase III activities in the extra-hepatic tissues alone are not sufficient to account for the observed rate of urea production.

Although CPSase III activities in extra-hepatic tissues of *M. abei* are low, the properties of this enzyme are quite similar to those in the Lake Magadi tilapia in that: (i) it has substantial activity in the absence of AGA, when glutamine (20 mmol l^{-1}) is used as the substrate; (ii) ammonia (5 mmol l^{-1}) appears to be as effective a nitrogen-donating substrate as glutamine; and (iii) there was little effect of UTP on enzyme activity with ammonia and also probably glutamine as the substrate. In the ureogenic Indian catfish *C. batrachus*, ammonia- and AGA-dependent CPSase activity and little inhibitory effect of UTP on CPSase were also observed in muscle, intestine and brain (Saha et al., 1999).

In the present study, the activities of none of the O-UC enzymes of the ammonia-exposed fish differred significantly from those of the controls, despite the two- to threefold increase in CPSase III mRNA expression in muscle and gill following exposure to ammonia. Similarly, in the gulf toadfish *Opsanus beta* following confinement stress, CPSase III mRNA expression in liver increased five- to tenfold with no change in CPSase III activity (Julsrud et al., 1998; Kong et al., 2000). In contrast, GSase activity and GSase mRNA expression in liver increased eight- and fivefold, respectively, during confinement (Kong et al., 2000). In the toadfish, GSase is considered to be a major factor contributing to increased ureogenesis (Anderson and Walsh, 1995; Walsh and Milligan, 1995; Kong et al., 2000). In *M. abei*, CPSase III activities in the muscle, skin and gill together are low but, as estimated above, the biosynthetic activity of GSase in the muscle (0.69 μmol h^{-1} g^{-1}) is sufficient to support the observed rate of urea production if glutamine is required for urea-related carbamoyl phosphate formation. Furthermore, the increase in glutamine concentration in muscle following exposure to ammonia may create favourable conditions for *in situ* urea synthesis. Indeed, in the toadfsh liver, an increase in glutamine concentration is known to result in an increase in AGA levels, which results in a much greater synergistic increase in CPSase activity (Julsrud et al., 1998).

The increased urea contents and excretion rate in the ammonia-exposed fish were accompanied by a twofold increase in citrulline levels in the muscle and whole fish, suggesting that sufficient carbamoyl phosphate is supplied to the O-UC. CPSase III activities measured *in vitro* may not reflect real activities *in vivo* because of losses of activities during the preparation of tissue extracts. Moreover, it is possible that urea formation in the muscle, skin and gill together exerts a synergistic effect, i.e. the total rate of urea production exceeds the rate deduced from the enzyme activity in each individual tissue through the cycling and transport of the substrates related to urea synthesis. However, this possibility needs to be tested more fully under defined conditions.

In ammoniotelic fishes, urea is considered to be produced mainly by uricolysis (Forster and Goldstein, 1969; Hayashi et al., 1989; Wood, 1993). To our knowledge, however, there has been little investigation on the metabolic pathway leading to *de novo* synthesis of purine in teleosts. The mitochondrial localization of GSase is well established for uricotelic liver, where its function is analogous to that of CPSase I or III in ureotelic species (Campbell, 1995). In a preliminary experiment, more than 90 % of GSase activity in the liver of *M. abei* was detected in the cytosol fraction along with lactate dehydrogenase (LDH) activity (K. Iwata, unpublished results). Wright (1993) reported that a three- to fivefold increase in the rate of urea excretion in the freshwater tilapia *Oreochromis niloticus* exposed to a high concentration of ammonia was attributable to an elevation in the activity of the uricolysis pathway (i.e. a significant elevation of allantoicase activity in the liver). In *M. abei*, there were no significant changes in uricase and allantoicase activities in the liver and muscle. Furthermore, uric acid levels in the muscle and whole body remained unchanged following exposure to ammonia. On the basis of these facts, it seems unlikely that urea synthesis *via* uricolysis is predominant in *M. abei*.

The gills and skin are known to be the major sites of urea extrusion (Morii et al., 1978; Sayer and Davenport, 1987; Wood, 1993). In the Lake Magadi tilapia, urea is assumed to be transported externally through chloride cells in the gills

(Maina, 1991). However, the present results reveal that both skin and gill are urea-producing sites in *M. abei*. At high environmental concentrations of ammonia, it is quite possible that ammonia enters the fish mainly across the gills and skin where urea is synthesized. If urea is produced in the gill and skin using external ammonia as the nitrogen-donating substrate, and then excreted at the same sites immediately after production, urea synthesis at these sites may result in futile energy expenditure rather than preventing ammonia from entering the body. Alternatively, ammonia entering across all body surfaces may be trapped primarily as amide-N in glutamine and subsequently converted to urea by the muscle, skin and gill for elimination at the gill and probably the skin. This route might operate when conditions for ammonia excretion from the fish into the environment are unfavourable. Further studies on the role of the O-UC in the gill and skin are needed.

We would like to express our cordial thanks to Dr P. M. Anderson, University of Minnesota, for giving us valuable information about CPSase III in fish. We wish to thank B. Wicks, University of British Columbia, for her valuable comments on the manuscript. This work was partly supported by Grant-in-Aid to K.I. (no. 11640679) and T.S. (no. 10740384) from the Japan Society for the promotion of Science and to T.S. (no.1203) from the Fisheries Agency of Japan.

References

Anderson, P. M. (1995). Urea cycle in fish: Molecular and mitochondrial studies. In *Fish Physiology*, vol. 14, *Cellular and Molecular Approaches to Fish Ionic Regulation* (ed. C. M. Wood and T. J. Shuttleworth), pp. 57–83. New York: Academic Press.

Anderson, P. M. and Walsh, P. J. (1995). Subcellular localization and biochemical properties of the enzymes of carbamoyl phosphate and urea synthesis in the batrachoidid fishes *Opsanus beta*, *Opsanus tau* and *Porichthys notatus*. *J. Exp. Biol.* **198**, 755–766.

Berl, S., Takagaki, G., Clarke, D. D. and Waelsch, H. (1962). Metabolic compartments *in vivo*, ammonia and glutamic acid metabolism in brain and liver. *J. Biol. Chem.* **237**, 2562–2569.

Blumenkrantz, N. and Asboe-Hansen, G. (1975). Differential assay for urea and citrulline, automated and manual procedures. *Clin. Biochem.* **8**, 293–297.

Brown, G. W. and Cohen, P. P. (1959). Comparative biochemistry of urea synthesis. I. Methods for quantitative assay of urea cycle enzymes in liver. *J. Biol. Chem.* **234**, 1769–1774.

Brown G. W., Jr, James, J., Henderson, R. J., Thomas, W. N., Robinson, R. O., Thompson, A. L., Brown, E. and Brown, S. G. (1966). Uricolytic enzyme in liver of the dipnoan *Protopterus aetiopicus*. *Science* **153**, 1653–1654.

Campbell, J. W. (1995). Excretory nitrogen metabolism in repetiles and birds. In *Nitrogen Metabolism and Excretion* (ed. P. J. Walsh and P. A. Wright), pp. 147–178. Boca Raton, FL: CRC Press.

Campbell, J. W., Bonner, S. P. and Lee, T. W. (1968). Enzymes of arginine and urea metabolism in invertebrates. In *Experiments in Physiology and Biochemistry*, vol. 1 (ed. G. A. Kerkut), pp. 1–23. London: Academic Press.

Ceriotti, G. and Spandrio, L. (1963). A spectrophotometric method for determination of urea. *Clin. Chim. Acta* **8**, 295–299.

Chadwick, T. D. and Wright, P. A. (1999). Nitrogen excretion and expression of urea cycle enzymes in the Atlantic cod (*Gadus morhua* L.): a comparison of early life stages with adults. *J. Exp. Biol.* **202**, 2653–2662.

Chamberlin, M. E., Glemet, H. C. and Ballantyne, J. S. (1991). Glutamine metabolism in a holostean (*Amia calva*) and teleost fish (*Salvelinus namaycush*). *Am. J. Physiol.* **260**, R159–R166.

Felskie, A. K., Anderson, P. M. and Wright, P. A. (1998). Expression and activity of carbamoyl phosphate synthetase III and ornithine urea cycle enzymes in various tissues of four fish species. *Comp. Biochem. Physiol.* **119**B, 355–364.

Forster, R. P. and Goldstein, L. (1969). Formation of excretory products. In *Fish Physiology*, vol. 1 (ed. W. S. Hoar and D. J. Randall), pp. 313–350. New York: Academic Press.

Ganda, O. P. and Ruderman, N. B. (1976). Muscle nitrogen metabolism in chronic hepatic insufficiency. *Metabolism* **25**, 427–435.

Hayashi, S., Fujiwara, S. and Noguchi, T. (1989). Degradation of uric acid in fish liver peroxisomes. Intraperoxisomal localization of hepatic allantoicase and purification of its peroxismal membrane-bound form. *J. Biol. Chem.* **264**, 3211–3215.

Helfman, G. S., Collette, B. B. and Facey, D. E. (1997). Taxonomy, phylogeny and evolution. In *The Diversity of Fishes* (ed. G. S. Helfman), pp. 264–265. Malden, UK: Blackwell Science.

Henry, R. J., Sobel, C. and Kim, J. (1957). A modified carbonate–phosphotungstate method for the determination of uric acid and comparison with the spectrophotometric uricase method. *Am. J. Clin. Path.* **28**, 152–160.

Iwata, K. (1988). Nitrogen metabolism in the mudskipper, *Periophthalmus cantonensis*: changes in free amino acids and related compounds in various tissues under conditions of ammonia loading, with special reference to its high ammonia tolerance. *Comp. Biochem. Physiol.* **91**A, 499–508.

Iwata, K. and Deguchi, M. (1995). Metabolic fate and distribution of ^{15}N-ammonia in the ammonotelic fish *Periophthalmus cantonensis*, following immersion in ^{15}N-ammonium sulfate: a long-term experiment. *Zool. Sci.* **12**, 175–184.

Iwata, K., Kakuta, I., Ikeda, M., Kimoto, S. and Wada, N. (1981). Nitrogen metabolism in the mudskipper, *Periophthalmus cantonensis*: a role of free amino acids in detoxification of ammonia produced during its terrestrial life. *Comp. Biochem. Physiol.* **68**A, 589–596.

Julsrud, E. A., Walsh, P. J. and Anderson, P. M. (1998). N-acetyl-glutamate and urea cycle in gulf toadfish (*Opsanus beta*) and other fish. *Arch. Biochem. Biophys.* **350**, 55–60.

Kong, H., Edberg, D. D., Korte, J. J., Salo, W. L., Wright, P. A. and Anderson, P. M. (1998). Nitrogen excretion and expression of carbamoyl-phosphate synthetase III activity and mRNA in extrahepatic tissues of largemouth bass (*Micropterus salmoides*). *Arch. Biochem. Biophys.* **350**, 157–168.

Kong, H., Kahatapitiya, N., Kingsley, K., Salo, W. L., Anderson, P. M., Wang, Y. S. and Walsh, P. J. (2000). Induction of carbamoyl phosphate synthetase III and glutamine synthetase mRNA during confinement stress in gulf toadfish (*Opsanus beta*). *J. Exp. Biol.* **203**, 311–320.

Korsgaard, B., Mommsen, T. P. and Wright, P. A. (1995). Nitrogen excretion in teleostean fish: Adaptive relationships to environment, ontogenesis and viviparity. In *Nitrogen Metabolism*

and Excretion (ed. P. J. Walsh and P. A. Wright), pp. 259–287. Boca Raton, FL: CRC Press.

Korte, J. J., Salo, W. L., Cabrera, V. M., Wright, P. A., Felskie, A. K. and Anderson, P. M. (1997). Expression of carbamoyl-phosphate synthetase III mRNA during the early stages of development and in muscle of adult rainbow trout (*Oncorhynchus mykiss*). *J. Biol. Chem*. **272**, 6270–6277.

Lindley, T. E., Scheiderer, C. L., Walsh, P. J., Wood, C. M., Bergman, H. L., Bergman, A. L., Laurent, P., Wilson, P. and Anderson, P. M. (1999). Muscle as the primary site of urea cycle enzyme activity in an alkaline lake-adapted tilapia, *Oreochromis alcalicus grahami*. *J. Biol. Chem.* **274**, 29858–29861.

Maina, J. N. (1991). A morphometric analysis of chloride cells in the gills of the teleosts *Oreochromis alcalicus* and *Oreochromis niloticus* and a description of presumptive urea-excreting cells in *O. alcalicus. J. Anat*. **175**, 131–145.

McKenzie, D. J. and Randall, D. J. (1990). Does *Amia calva* aestivate? *Fish Physiol. Biochem*. **8**, 147–158.

Mommsen, T. P. and Walsh, P. J. (1991). Urea synthesis in fishes: evolutionary and biochemical perspectives. In *Biochemistry and Molecular Biology of Fishes*, vol. 1 (ed. P. W. Hochachka and T. P. Mommsen), pp. 137–163. New York: Elsevier Science.

Morii, H., Nishikata, K. and Tamura, O. (1978). Nitrogen excretion of mudskipper fish *Periophthalmus cantonensis* and *Boleophthalmus pectinirostris* in water and on land. *Comp. Biochem. Physiol*. **60**A, 189–193.

Pierson, D. (1980). A rapid colorimetric assay for carbamyl phosphate synthetase I. *J. Biochem. Biophys. Meth*. **3**, 31–37.

Rahmatullah, M. and Boyde, T. R. C. (1980). Improvements in the determination of urea using diacetyl monoxime; methods with and without deproteinisation. *Clin. Chim. Acta* **107**, 3–9.

Randall, D. J., Wood, C. M., Perry, S. F., Bergman, H., Maloiy, G. M. O., Mommsen, T. P. and Wright, P. A. (1989). Urea excretion as a strategy for survival in a fish living in a very alkaline environment. *Nature* **337**, 165–166.

Ruderman, N. B. and Lund, P. (1972). Amino acid metabolism in skeletal muscle: Regulation of glutamine and alanine release in the perfused rat hindquarter. *Israel J. Med. Sci*. **8**, 295–302.

Saha, N., Das, L. and Dutta, S. (1999). Types of carbamyl phosphate synthetases and subcellular localization of urea cycle and related enzymes in air-breathing walking catfish, *Clarias batrachus*. *J. Exp. Zool*. **283**, 121–130.

Saha, N. and Ratha, B. K. (1987). Active ureogenesis in a freshwater air-breathing teleost, *Heteropneustes fossilis*. *J. Exp. Zool*. **241**, 137–141.

Saha, N. and Ratha, B. K. (1994). Induction of ornithine–urea cycle in a freshwater teleost, *Heteropneustes fossilis*, exposed to high concentrations of ammonium chloride. *Comp. Biochem. Physiol.* **108**B, 315–325.

Sayer, M. D. J. and Davenport, J. (1987). The relative importance of the gills to ammonia and urea excretion in five seawater and one freshwater teleost species. *J. Fish Biol*. **31**, 561–570.

Shankar, R. A. and Anderson, P. M. (1985). Purification and properties of glutamine synthetase from liver of *Squalus acanthias*. *Arch. Biochem. Biophys.* **239**, 248–259.

Solorzano, L. (1969). Determination of ammonia in natural waters by the phenylhypochlorite method. *Limnol. Oceanograph*. **14**, 799–801.

Takagaki, G., Berl, S., Clarke, D. D., Purpura, D. P. and Waelsch, H. (1961). Glutamic acid metabolism in brain and liver during infusion with ammonia labelled with nitrogen-15. *Nature* **189**, 326.

Terjesen, B. F., Ronnestad, I. R., Norberg, B. and Anderson, P. M. (2000). Detection and basic properties of carbamoyl phosphate synthetase III during teleost ontogeny: a case study in the Atlantic halibut (*Hippoglossus hippoglossus* L.). *Comp. Biochem. Physiol.* **126**B, 521–535.

Van Waarde, A. and Kesbeke, F. (1982). Nitrogen metabolism in goldfish, *Carassius auratus* (L.). Activities of amidases, and amide synthetases in goldfish tissues. *Comp. Biochem. Physiol.* **71**, 599–603.

Walsh, J. P., Danulat, E. and Mommsen, T. P. (1990). Variation in urea excretion in the gulf toadfish *Opsanus beta*. *Mar. Biol.* **106**, 323–328.

Walsh, P. J. and Milligan, C. L. (1995). Effects of feeding and confinement on nitrogen metabolism and excretion in the gulf toadfish *Opsanus beta. Mar. Biol*. **106**, 323–328.

Walton, M. J. and Cowey, C. B. (1977). Aspects of ammoniogenesis in rainbow trout, *Salmo gairdneri*. *Comp. Biochem. Physiol*. **57**, 143–149.

Webb, J. T. and Brown, G. W., Jr (1976). Some properties and occurrence of glutamine synthetase in fish. *Comp. Biochem. Physiol.* **54**B, 171–175.

Wood, C. M. (1993). Ammonia and urea metabolism and excretion. In *The Physiology of Fishes* (ed. D. H. Evans), pp. 379–425. Boca Raton, FL: CRC Press.

Wright, P. A. (1993). Nitrogen excretion and enzyme pathways for ureagenesis in freshwater tilapia (*Oreochromis niloticus*). *Physiol. Zool*. **66**, 881–901.

Wright, P. A., Felskie, A. and Anderson, P. M. (1995). Induction of ornithine–urea cycle enzymes and nitrogen metabolism in rainbow trout (*Oncorhynchus mykiss*) during early life stages. *J. Exp. Biol.* **198**, 127–135.